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13. ABSTRACT *(Maximum 200 words)*

Bcl-2 is a potent inhibitor of apoptosis induced by a variety of insults. Bcl-2 is highly expressed in breast cancer and its expression is related to chemotherapy and radiation therapy resistance. Recent studies suggest that ECM plays a crucial role in apoptosis regulation in breast epithelial cells. In this report, we investigated whether bcl-2 inhibition of apoptosis involves regulation of ECM molecules such as MMPs and TIMPs. We reported that bcl-2 overexpression upregulates TIMP-1 and downregulates MMP-9 in breast epithelial cell lines (MCF10A, MCF10ATG3B and MCF-7), while it has no effect on TIMP-2 expression. We demonstrated that TIMP-1 inhibits cell death induced by hydrogen peroxide, Adriamycin. In addition, TIMP-1 overexpression inhibits apoptosis following the loss of cell adhesion (anoikis) in MCF10A cells suggesting that the anti-apoptotic activity of TIMP-1 does not depend on its ability to stabilize cell-matrix interactions. We also showed that TIMP-1 overexpression is associated with a constitutive activation of the focal adhesion kinase, a signaling molecule known to be critical for the cell survival pathway.

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FOREWORD

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Gregory L 7-12-99

PI - Signature Date

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INTRODUCTION

Bcl-2 is a major anti-apoptotic molecule which is localized to the outer mitochondral membrane, endoplasmic reticulum and nuclear envelop (1). Bcl-2 prevents cytochrome C release and inhibits the activation of caspases, a group of cysteine proteases that initiate the apoptotic process (2, 3). However, recent studies suggest pleiotropic roles for bcl-2 in apoptosis regulation including modulation of Ca^{2+} homeostasis, transcription factors, and signaling kinases (4-6). Recent studies suggest that numerous cellular activities are influenced by the extracellular matrix (ECM). Cell-matrix interactions have been shown to greatly influence cell survival, and withdrawal of anchorage-dependent cells from their association with ECM results in apoptotic cell death (7, 8). For example, apoptosis of secretory epithelial cells during involution of the mammary gland after lactation is accompanied by proteolytic degradation of the gland basement membrane (9). Consistently, overexpression of stromelysin-1, a member of the matrix metalloproteinase (MMP) family of ECM-degrading enzymes, was shown to induce apoptosis in mammary epithelial cells *in vitro* and in transgenic mice (10), possibly due to its effect on ECM integrity. When stromelysin-1 transgenic mice were crossed with mice overexpressing tissue inhibitor of metalloproteinase-1 (TIMP-1), a natural MMP inhibitor, apoptosis was significantly inhibited (11), suggesting a role for MMPs and TIMPs in ECM-regulation of cell survival. Although the precise mechanisms by which TIMPs control cell survival remain undefined, their effect may be mediated by their ability to regulate proteolysis of both ECM components and other biologically relevant molecules. Indeed, TIMP-3 induction of apoptosis may be mediated by its inhibition of MMPs, resulting in stabilization of tumor necrosis factor (TNF)-α receptors on the cell surface (12). However, apoptosis regulation by TIMPs may not be only related to their anti-proteolytic activity. For example, although TIMP-1 and TIMP-2 inhibition of MMP enzymatic activity is interchangeable, apoptosis regulation by these inhibitors was shown to be tissue-specific, suggesting that mechanisms other than inhibition of enzymatic activity may be involved (13-15). Both TIMP-1 and TIMP-2 were also shown to regulate cell proliferation, suggesting that TIMP effects on cell survival may be mediated by yet undefined signaling pathways independent of their anti-proteolytic activity (16-19). Indeed, TIMP-1 and TIMP-2 have been shown to stimulate tyrosine kinase and mitogen-activated protein kinase (MAPK) activity in human osteosarcoma cell line MG-63 (20). As a model to study the anti-apoptotic effects of bcl-2 in breast epithelial (BE) cells, we established stable transfectants of various BE cell lines overexpressing bcl-2. In this report, we investigated whether bcl-2 inhibition of apoptosis involves regulation of ECM molecules. Here we report that bcl-2 overexpression results in induction of TIMP-1 expression and that TIMP-1 expression in the absence of bcl-2 overexpression efficiently inhibits apoptosis. We present evidence that TIMP-1 inhibition of apoptosis involves modulation of signaling pathways, including activation of focal adhesion kinase (FAK).

BODY OF RESEARCH

This annual summary covers research work from July, 1998- July, 1999.

TIMP-1 enhance breast epithelial survival

Previous studies suggested an anti-apoptotic role for TIMP-1 (13, 14). Since bcl-2 expression results in a high level of TIMP-1 expression in breast epithelial cells (last year's report), we examined whether TIMP-1 plays a direct role in the survival of MCF10A cells after exposure to apoptotic agents. To this end, MCF10A cells were exposed to H_2O_2 in the presence or absence of exogenously added TIMP-1. Preliminary dose-dependence experiments showed that H_2O_2 (250 to 500 μM) induced cell death, mostly by apoptosis as determined by nuclear morphological analysis (21). As shown in Table 1, in the absence of TIMP-1, approximately 12% of cells remained viable following 48 hours of H_2O_2 treatment. In the presence of TIMP-1 (500 ng/ml) cell survival increased to 23%. In contrast, similar amounts of TIMP-2 had no effect on H_20_2-induced cell death in MCF10A cells (data not shown).

To eliminate the possibility that TIMP-1-enhanced cell survival following H_2O_2 treatment was due to TIMP-1 mitogenic activity, MCF10A cells were incubated with ^{3}H-thymidine for 48 hours, followed by serum-free culture for 24 hours and then examined for survival after H_2O_2 treatment in the presence or absence of TIMP-1. These studies demonstrated similar results with 1.5-2 fold more ^{3}H-thymidine associated in the cells treated in the presence of TIMP-1, compared to that in H_20_2-treated cells without TIMP-1 (data not shown,). This indicated that the increased cell survival rate following H_2O_2 treatment in the presence of TIMP-1 results from an effect of TIMP-1 on cell survival, and not on proliferation.

To further investigate the role of TIMP-1 in the regulation of apoptosis in human breast epithelial cells, we introduced a TIMP-1-expression vector into MCF10A cells. TIMP-transfected MCF10A clones were isolated and the level of TIMP-1 expression was determined by immunoblot analysis. As shown in Fig. 1, both intracellular and extracellular levels of TIMP-1 increased 3-6 folds in the TIMP-1-transfected MCF10A cells. The TIMP-1 expression levels in the TIMP-1-transfected MCF10A were comparable to those observed in the MCF10A cells overexpressing bcl-2. We next investigated whether the endogenous TIMP-1 could enhance cell survival against apoptotic stimuli including H_2O_2, Adriamycin. In addition, we compared the TIMP-1 overexpressing cells with the bcl-2 overexpressing cells. These studies demonstrated a similar rate of survival after these treatments in MCF10A cells overexpressing TIMP-1 or bcl-2 (Fig. 2 A and B). TIMP-1 inhibition of apoptosis was further confirmed by nuclear morphological analysis (Fig. 3). Whereas the control cells showed fragmented nuclei that were consistent with nuclear morphological changes in apoptotic cells (21), no significant changes in nuclear morphology could be observed in either the TIMP-1- or the bcl-2 overexpressing cells. It should be mentioned that TIMP-1 overexpression had no effect on the basal levels of bcl-2 expression in the TIMP-1-transfected clones, suggesting that its effect on apoptosis is independent of the bcl-2 expression level (Fig. 4).

TIMP-1 inhibition of apoptosis does not depend on its ability to stabilize cell-substrates or cell-cell interactions.

Survival of epithelial cells is dependent on their interaction with the ECM (7, 22). Following loss of cell anchorage, epithelial cells undergo anoikis, the process of apoptosis caused by loss of substrate adhesion (7, 22). TIMP-1 inhibition of apoptosis may result from its ability to stabilize cell-ECM interactions by inhibiting MMPs. If so, we hypothesized that TIMP-1 would not protect against apoptosis induced by loss of cell-substrate interactions. To induce anoikis, the control and the MCF10A cells overexpressing TIMP-1 or bcl-2 were cultured in dishes coated with polyHEMA to prevent cell adhesion. After twenty-four hours, cell survival was determined by trypan blue exclusion assay. These studies showed that <20% of the control MCF10A cells remained viable in the polyHEMA-coated dishes consistent with induction of anoikis, as previously described (7). In contrast, ~ 80% of the bcl-2 or TIMP-1 overexpressing cells remained viable under the same conditions (Fig. 5A). Cleavage of poly (ADP-ribose) polymerase (PARP) is an early event in the process of apoptosis resulting from the activation of caspase/Ced-3 family members (23). We therefore examined the PARP cleavage in TIMP-1-overexpressing cells cultured in polyHEMA-coated dishes. As shown in Fig. 5B, apoptosis-specific proteolytic cleavage of PARP (85-kDa fragment) was readily detected in suspension cultures of the control cells, whereas it was significantly inhibited in the bcl-2- or TIMP-1-overexpressing cells. Anchorage-independent survival of the control MCF10A, bcl-2- or TIMP-1-overexpressing cells was also evaluated by culturing cells in soft agar. As shown in Fig. 6, > 80% of bcl-2- or TIMP-1-overexpressing cells remained viable even after 7 days of culture in soft agar, while < 20% of the control MCF10A cells survived. Thus, both bcl-2 and TIMP-1 can prevent anoikis in MCF10A cells. Taken together, these studies suggest that TIMP-1 inhibits caspase-mediated apoptosis independent of its ability to stabilize cell-substrate or cell-cell interactions.

Overexpression of TIMP-1 is associated with constitutive activation of the focal adhesion kinase (FAK) in an anchorage-independent manner.

Increasing evidence indicates that interactions of integrins with the ECM transduce biochemical signals that are mediated, in part, by the activation of the focal adhesion kinase (FAK) (22, 24-26). Neutralizing antibodies against integrins induce cell detachment followed by anoikis in epithelial cells, suggesting a role for integrin signaling in the regulation of anoikis (22, 27). Constitutively activated forms of FAK (tyrosine phosphorylated form) play a role in protection against anoikis (28) and free radical-induced cell death (29), suggesting that FAK activity is critical for cell survival. Therefore, we examined whether the TIMP-1 anti-apoptotic activity involved the modulation of FAK activity. The expression levels of FAK were not altered by TIMP-1 overexpression, as determined by immunoblot analysis using an anti-FAK mAb (Fig. 7A). We next examined whether TIMP-1 modulates FAK activity. To this end, the FAK protein was immunoprecipitated with an anti-FAK mAb and the active form was detected by immunoblot analysis using an anti-phosphotyrosine antibody. As shown in Fig. 7B, FAK is more efficiently activated in TIMP-1-overexpressing cells than in the control cells. Since studies showed that FAK activation requires cell anchorage (24-26), we asked whether TIMP-1 upregulation of FAK activation also required cell anchorage. To this end, we cultured the control and TIMP-1-overexpressing cells in suspension for 12 hours and examined tyrosine-phosphorylated FAK. As shown in Fig. 7C, TIMP-1

constitutively activated FAK regardless of cell anchorage. This suggests that TIMP-1 regulates apoptosis through constitutive activation of cell survival signaling pathways.

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Appendix 1

A LIST OF KEY RESEARCH ACCOMPLISHMENTS:

1. TIMP-1 WAS FOUND TO ENHANCE BREAST EPITHELIAL CELL SURVIVAL.
2. TIMP-1 INHIBITION OF APOPTOSIS DOES NOT DEPEND ON ITS ABILITY TO STABILIZE CELL-MATRIX OR CELL-CELL INTERACTION.
3. OVEREXPRESSION OF TIMP-1 IS ASSOCIATED WITH CONSTITUTIVE ACTIVATION OF THE FOCAL ADHESION KINASE IN AN ANCHORAGE-INDEPENDENT MANNER.

A LIST OF OUTCOMES:

1. A Ph.D. DEGREE WAS EARNED BY THE PRINCIPAL INVESTIGATOR.
2. A POSTDOCTORAL POSITION WAS OFFERED TO THE PRINCIPAL INVESTIGATOR BY THE DEPARTMENT OF PATHOLOGY, WAYNE STATE UNIVERSITY SCHOOL OF MEDICINE.

TABLE 1. TIMP-1 enhance survival in MCF10A cells

Culture Dishes	1	2	3	Mean ±S.D.	% of Cell Survival
	Cell Number X 10^5				
Serum-free Medium	7.2	8.8	10.1	8.7±1.453	100%
TIMP-1 only	7.7	9.6	8.3	8.53± 0.9713	97.7%
Hydrogen Peroxide	1.0	0.9	1.3	1.067± 0.208	12.3% *
TIMP-1 and Hydrogen Peroxide	1.7	2.3	1.9	1.967± 0.306	22.6% *

*The p value is 0.02, which is less than 0.05. This means the difference is significant between hydrogen peroxide treatment group and TIMP-1 plus hydrogen peroxide treatment group.
Culture dishes 1-3 mean three different culture dishes. The numbers below indicate cell number times one hundred thousand.

Appendix 3

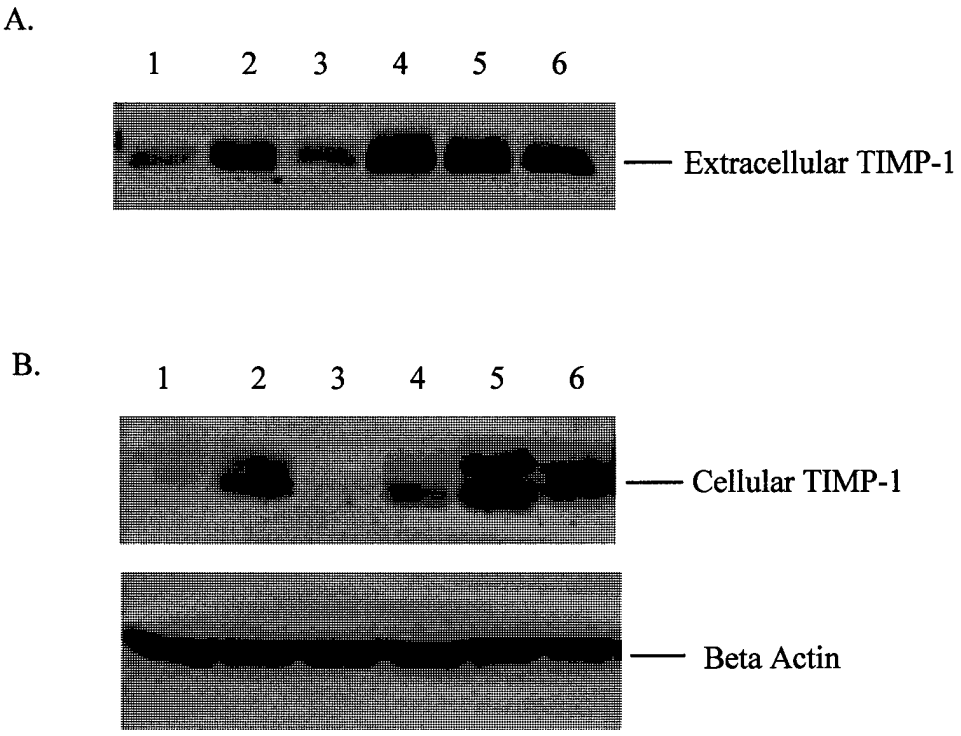
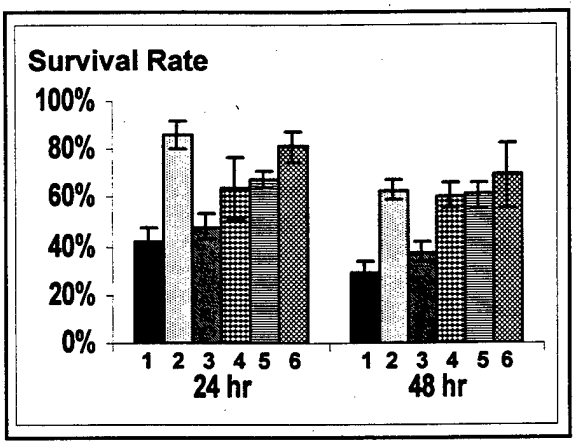


Figure 1. Western blot analysis of TIMP-1 protein from conditioned media and cell lysates of MCF10Aneo control, Bcl-2 and TIMP-1 overexpressing cells.
A: Equal amount of protein of conditioned media from each cell line was analyzed using an anti-TIMP-1 antibody. The figure shows the secreted TIMP-1 levels of MCF10A neo control clone #1 and #2 (lanes 1 and 3)respectively, Bcl-2 over-expressing MCF10A clone #2 (lane 2), and TIMP-1 overexpressing clones # 3, #29 and pooled population (lanes 4, 5 and 6)respectively.
B: The upper panel shows TIMP-1 protein levels from the cell lysates of MCF10A neo control clone #1 and #2 (lanes1 and 3)respectively, Bcl-2 overexpressing MCF10A clone #2 (lane 2), and TIMP-1 overexpressing clones #3, #29 and pooled population, (lanes 4, 5 and 6) respectively. The lower panel shows the beta actin levels of the corresponding lanes of the upper panel.

Appendix 4

A.



B.

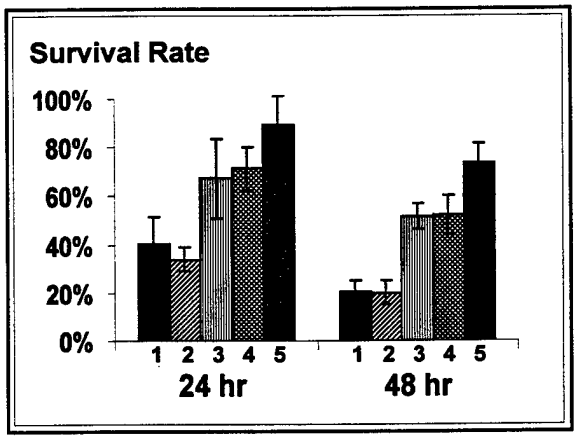


Figure 2. TIMP-1 protects MCF10A cells against hydrogen peroxide and Adriamycin induced cell death.

A: MCF10Acontrol, TIMP-1 and bcl-2 overexpressing MCF10A cells were treated with 500 uM hydrogen peroxide. All cell lines were prepared in triplicate. The cells were counted at day 1 (0 hour) as cell number control and counted at 24 hours, 48 hours after hydrogen peroxide treatment. Bar one and three represent MCF10Aneo control #1 and #2 respectively. Bar two represents bcl-2 overexpressing cells. The last three bars represent TIMP-1 overexpressing clones #3, #29 and pooled population.

B: Each cell line was plated in triplicate.The MCF10Aneo control and TIMP-1 overexpressing cells were treated with 0.5 ug/ml of adriamycin. The cell lines were then counted at day 1 (0 hour) before addition of adriamycin as cell number control for the respective cell lines. After addition of 0.5 ug/ml adriamycin, the cells were counted at 24 hour and 48 hours. The first two bars represent MCF10Aneo clone #1 and #2. The last three bars represent TIMP-1 overexpressing MCF10A clone # 3, #29, and pooled populations.

Appendix 5

MCF10A TIMP-1 Control Cells TIMP-1 Overexpressing MCF10A

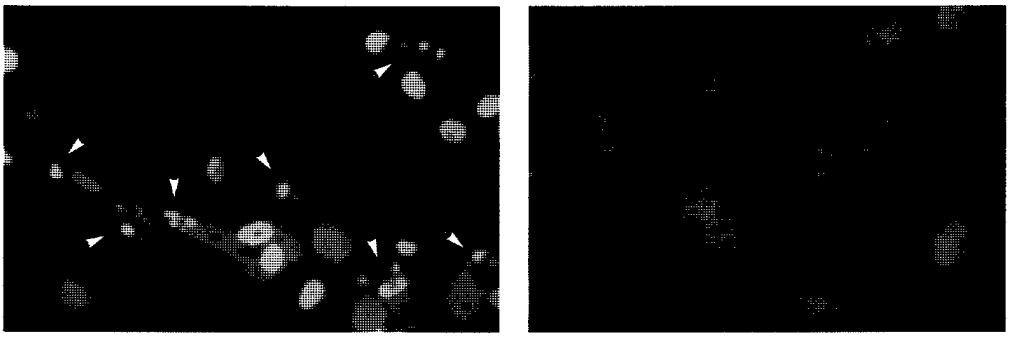


Figure 3. Hydrogen peroxide induction of apoptosis in MCF10A TIMP-1 control and TIMP-1 overexpressing MCF10A cells.

Nuclear morphologies of the MCF10A TIMP-1 control and TIMP-1 overexpressing MCF10A cells stained with bisBenzimide 24 hours after treatment with 500 uM hydrogen peroxide. Arrows in the picture indicate fragmented nucleus of apoptotic cells.

Appendix 6

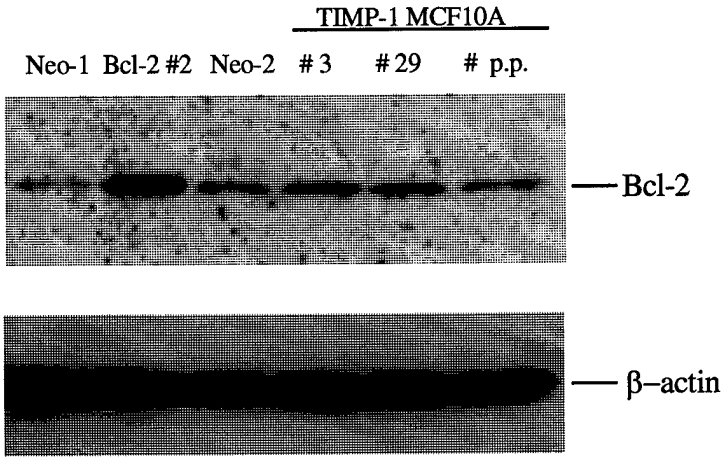
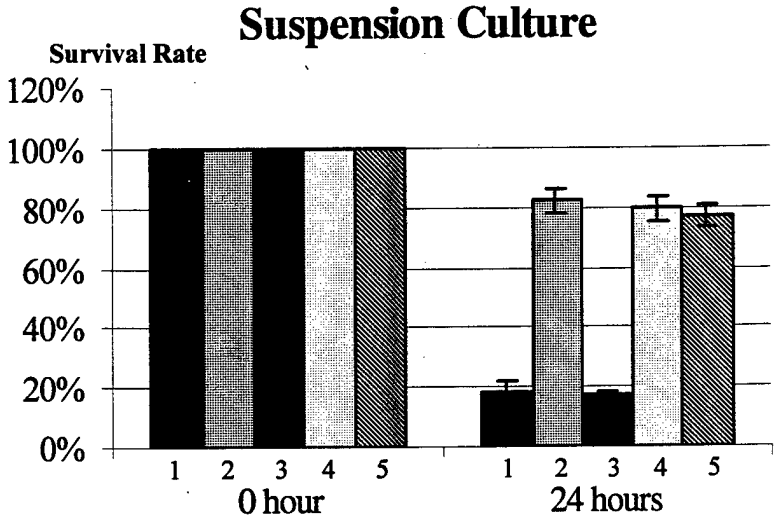


Figure 4. Western blot analysis of Bcl-2 protein from the cell lysates of MCF10A control, Bcl-2 and TIMP-1 overexpressing MCF10A cells.
The upper panel shows Bcl-2 levels of MCF10A neo control clone #1 and #2 (lanes 1 and 3) respectively, Bcl-2 overexpressing clone #2 (lane 2), and TIMP-1 overexpressing clones #3, #29 and pooled population (lanes 4, 5 and 6) respectively. The lower panel shows the beta actin levels of the upper panel.

Appendix 7

A.



B.

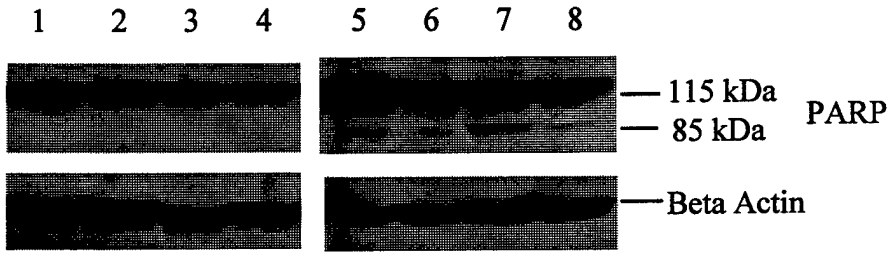


Figure 5. Induction of anoikis in MCF10A cells.
A: MCF10Aneo control cells, Bcl-2 and TIMP-1 overexpressing MCF10A cells were plated on polyHEMA coated petri dishes. After 24 hours, cells were counted using trypan blue exclusion assay. Both viable cells and dead cells were counted. Bars 1 and 3 represent MCF10Aneo control clone #1 and #2 respectively. Bar 2 represents Bcl-2 overexpressing MCF10A clone #2. Bars 4 and 5 represent TIMP-1 overexpressing clones #3 and #29 respectively. Zero hour indicates that cells grow under normal condition and the survival rates were assumed to be 100% for each cell line population.
B: Western blot analysis of PARP from MCF10A cells. Lane 1, 2 ,3 and 4 represent MCF10A cells growing under normal conditions. Lanes 1, and 3 represent MCF10A neo control clone #1 and #2 respectively. Lane 2 and 4 represent Bcl-2 overexpressing clone #2 and TIMP-1 overexpressing clone #3 lines respectively. Lanes 5, 6, 7 and 8 represents MCF10A cells plated on polyHEMA. Lanes 5 and 7 represent MCF10A neo control clone #1 and #2. Lanes 6 and 8 represent Bcl-2 overexpressing clone #2 and TIMP-1 overexpressing clone #3 respectively. The 85 kDa PARP cleavage bands in the upper panel indicate early signs of apoptosis. The lower panel shows the beta actin levels of their respective blots.

Appendix 8

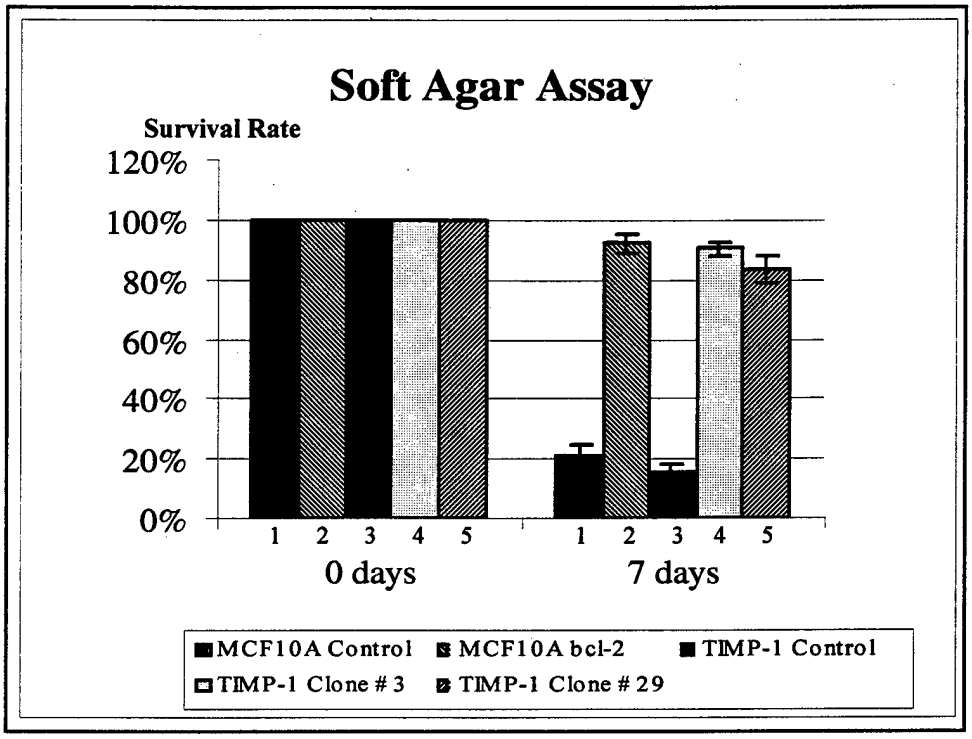


Figure 6. Soft agar assays of MCF10Aneo control, Bcl-2 and TIMP-1 over-expressing cell populations.
MCF10Aneo control, Bcl-2 and TIMP-1 overexpressing MCF10A cells were cultured on soft agar for one week, then exposed to trypan blue staining. Viable cells and dead cells were counted separately. At least five microscopic fields of viable cells to total cells ratios were calculated for each cell population. Bars 1 and 3 represent MCF10A neo control clone #1 and #2. Bar 2 represents Bcl-2 overexpressing clone #2. Bars 4 and 5 represent TIMP-1 overexpressing clones #3 and #29. Zero hour indicate that the cells grow under normal condition with little cell death. The survival rates under normal culture conditions were assumed to be 100% for each cell population.

Appendix 9

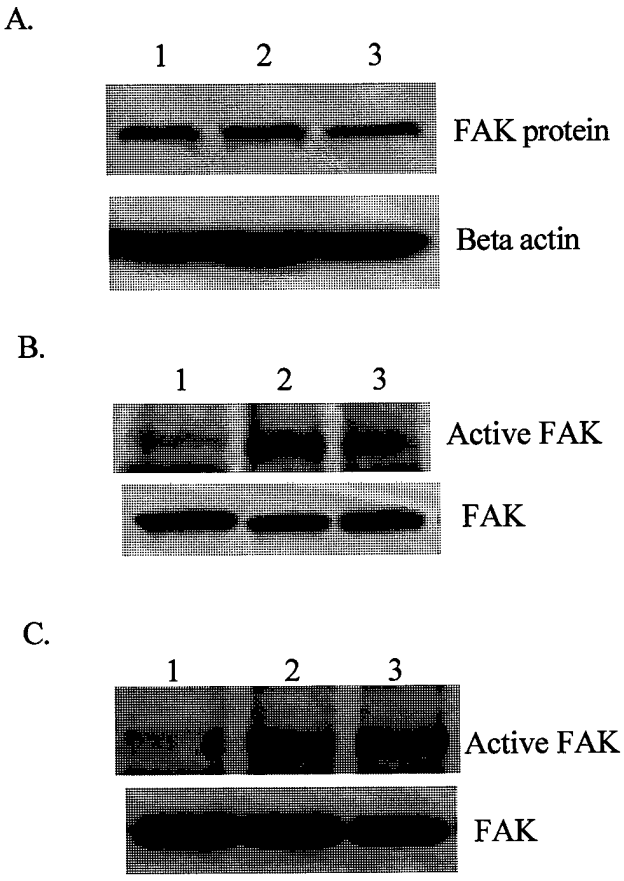


Figure 7. Western blot analysis and immunoprecipitation assay for FAK.
A: Cells were lysed in cell lysis buffer. Equal amount of protein from each cell line was analyzed for FAK expression. The upper panel shows FAK levels of MCF10A neo control (lane 1) and TIMP-1overexpressing clones #3 and pooled population (lanes 2 and 3) respectively. The lower panel shows the beta actin levels of the same blot.
B: Immunoprecipitation assay of FAK phosphorylation under normal growing condition. Equal amount of protein from each cell line was immunoprecipitated with an anti-human FAK monoclonal antibody. The active FAK was recognized with an anti-phosphotyrosine antibody. Lane 1 represents MCF10A neo control cells, while lanes 2 and 3 represents TIMP-1 overexpressing clones # 3 and pooled populations respectively. The lower panel represents the same blot reprobed with an anti-FAK antibody
C: Immunoprecipitation assay for FAK phosphorylation of MCF10Aneo control and TIMP-1 overexpressing cells on polyHEMA. Cells were plated on polyHEMA coated culture dishes for 12 hours. Equal amount of protein from each cell line was immunoprecipitated using an anti-human FAK antibody. The active FAK was recognized with an anti-phosphotyrosine antibody. Lane 1 represent MCF10Aneo control cells. Lanes 2 and 3 represents TIMP-1 overexpressing clones #3 and pooled populations respectively. The lower panel represents the same blot reprobed with an anti-FAK antibody.

GRADUATE SCHOOL WAYNE STATE UNIVERSITY

Appendix 10

FINAL REPORT
DISSERTATION PUBLIC LECTURE PRESENTATION - DEFENSE

(see reverse for instructions)

Name of Candidate: Gangyong Li

Major: Pathology Advisor: Dr. Hyeong-Reh Choi Kim

Dissertation Title: A novel role of BCL-2 in the regulation of apoptosis through induction of TIMP-1 in human breast epithelial cells.

DISSERTATION APPROVAL

"I have read and approved the content of this dissertation, for a Public Lecture Presentation - Defense"

Dissertation Committee Names: (please type)	Dissertation Committee Signatures:
Dr. Hyeong-Reh Choi Kim Advisor	Hyeong-Reh Choi Kim
Dr. Tuan-Huey Kuo	Tuan-Huey Kuo
Dr. Roberta G. Pourcho	Roberta G. Pourcho
Dr. Rafael A. Fridman	Fridman

Edith Warner
Signature: Format and appearance approved by the Graduate School (Pending Corrections)

ARRANGEMENTS FOR PUBLIC LECTURE PRESENTATION - DEFENSE

DATE: Fri. 4/23/99 TIME: 2:30 P.M. PLACE: 9364 Scott Hall

Departmental Graduate Committee Chairperson's Signature: C.H. Kim

COMMITTEE REPORT ON DISSERTATION PUBLIC LECTURE PRESENTATION - DEFENSE:

After review of the dissertation, and on the basis of the lecture presentation - defense, the Examining Committee certifies that the Candidate:

(X) Satisfactorily passed the Ph.D. Public Lecture Presentation - Defense.

( ) Did not satisfactorily pass the Ph.D. Public Lecture Presentation - Defense.

Date of Presentation: 4-23-99

Examining Committee Members Signatures:

Recommendations:

Tuan-Huey Kuo
Roberta G. Pourcho
Fridman

Frederic S. Pearson
Signature: Graduate Examiner

Appendix 11

ABSTRCT

A NOVEL ROLE FOR BCL-2 IN THE REGULATION OF APOPTOSIS THROUGH INDUCTION OF TIMP-1 IN HUMAN BREAST EPITHELIAL CELLS

by

GANGYONG LI

December, 1999

Advisor: Hyeong-Reh C. Kim, Ph.D.

Major: Pathology

Degree: Doctor of Philosophy

The product of oncogene bcl-2 is a potent inhibitor of apoptosis induced by a wide variety of stimuli. Bcl-2 is highly expressed in some malignant tumors including breast cancer and its expression is related to chemotherapy and radiation therapy resistance. Increasing evidence suggests that the extracellular matrix (ECM) plays a critical role in the regulation of apoptosis and serves as a survival factor for epithelial and endothelial cells. In the present study, we investigated whether Bcl-2 inhibition of apoptosis involved regulation of ECM remodeling enzymes and their inhibitors using three human breast epithelial cell lines: MCF10A, an immortalized "normal" breast epithelial cell line; MCF10AneoT.TG3B, a pre-neoplastic cell line derived from MCF10A; and MCF-7, a breast adenocarcinoma cell line. Our studies showed that Bcl-2 down-regulated MMP-9 expression, and up-regulated TIMP-1 expression, while it had no effect on TIMP-2 expression. We then investigated whether Bcl-2 induction of TIMP-1 is related to its anti-apoptotic activity. To this end, we tested whether recombinant TIMP-1 protein and/or TIMP-1 overexpression protect human breast epithelial cells from apoptosis. Our data showed that TIMP-1 inhibited apoptosis

induced by a variety of stimuli including hydrogen peroxide, adriamycin and irradiation as effectively as Bcl-2. Interestingly, TIMP-1 overexpression resulted in MMP-9 down-regulation, suggesting that Bcl-2 down-regulation of MMP-9 may result from TIMP-1 induction. We then examined whether TIMP-1 inhibited anoikis, a specific form of apoptosis induced by loss of cell-matrix interactions. Surprisingly, we found that TIMP-1 protected MCF10A cells against anoikis as effectively as Bcl-2. This suggests that TIMP-1 inhibition of apoptosis does not depend on its ability to stabilize cell-matrix interactions, leading us to hypothesize that TIMP-1 regulation of apoptosis is independent of its ability to inhibit MMPs.

Mounting evidence showed that interactions of integrins (a group of heterodimeric cell surface receptors) with ECM transduce biochemical signals, including activation of focal adhesion kinase (FAK), which is essential for cell survival and inhibition of apoptosis. Thus, we studied the role of TIMP-1 in the regulation of the integrin signaling pathway. We demonstrated that TIMP-1 constitutively activated focal adhesion kinase in a cell anchorage independent manner in MCF10A cells. Taken together, our studies showed TIMP-1 may be a downstream mediator of Bcl-2, and TIMP-1 inhibition of apoptosis results from its ability to modulate cell survival signaling, rather than its ability to stabilize cell-matrix interactions.

Appendix 12

Wayne State University
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June 4, 1999

Gangyong Li, Ph.D.
615 West Hancock
Apt. 203
Detroit, MI 48201

Dear Dr. Li:

On behalf of the School of Medicine and the Department of Pathology, we are pleased to offer you an appointment as a Post Doctoral Research Fellow. This appointment is to begin on or about May 31, 1999, depending on when pre-employment conditions are met and extend through April 30, 2000. This is a non-tenure track assignment and carries no presumption of continuing tenure. This assignment is subject to the pleasure of the President or his/her designee and contingent on the availability of funding. Furthermore, if you are in the United States on a non-immigrant visa status which allows employment at Wayne State University, this assignment terminates with the expiration of your visa status. Your salary will be $28,000 per 12 month year. The terms of this agreement may not be modified or altered by any oral statements or representations. The agreement may be modified only in writing, signed by a University official as authorized by Executive Order 85-1.

You will be responsible to the Dean and by his/her delegation to John D. Crissman, M.D. In this case, you will report to Hyeong-Reh Kim, Ph.D. Your duties, subject to periodic review, will include:

- Perform promoter and apoptosis assays.
- Perform work related to molecular biology.
- Perform work related to cell biology.
- Assist in the preparation of manuscripts.
- Other duties as assigned.

United States Law requires that you must provide evidence of identity and employment authorization. In order to do that, you must appear in person to complete the U.S. government Form I-9, and to provide the documents that the law requires. The attached form and list of documents is provided to assist you in completing this process.

Employment eligibility verification must take place before you begin service to the University. Arrangements may be made to satisfy the Form I-9 verification process by appearing in person at the School of Medicine Personnel Office, Room 1248 Scott Hall or the University Employment Services Office, 5700 Cass, Suite 1900 AAB, between 9:00 and 4:00 (if you are a United States citizen or permanent resident) or at the University International Services Office at 5460 Cass between 8:30 and 5:00 (if you are a non-immigrant alien). (If you are non-immigrant alien you will need to update this process with the International Services Office prior to each assignment. If you are a citizen or permanent resident of the United States and if you have completed the I-9 process at Wayne State University within the last three years, you will have already completed this requirement).

If you are not a citizen or permanent resident of the United States, this agreement is contingent upon your holding and maintaining approved employment authorization. If you are on a non-immigrant visa and work prior to or beyond approved employment authorization, the work will be considered "unauthorized employment" by the Immigration and Naturalization Service and a violation of status.

It must be understood that at least a major portion of your salary is being derived from grant/contract funds; that is, your appointment is specifically conditioned on availability of funding. The continuation of the assignment is dependent upon your satisfactory performance and upon the continuation of the funding. Should the funding be terminated or reduced, there is no direct, indirect or implied commitment by the Principal Investigator, the Department, the School, or Wayne State University to continue your employment (salary and fringe benefits) beyond the cessation of, or reduction in, the funding.

This position carries a substantial and valuable body of fringe benefits. As a 12 month appointee, you will accrue vacation. University policy does not permit payoff for accumulated but unused vacation days. Therefore, vacation days need to be used prior to separation; otherwise, they will be forfeited.

The personnel policies that apply to your classification are covered in the Wayne State University Personnel Manual for All Non-Represented Employees (Non-Rep Manual). A copy of the current Non-Rep Manual is enclosed. The Manual is updated as required with all changes and revised sections mailed directly to your university address. If a copy of the Non-Rep Manual is not enclosed with the letter, please contact the School of Medicine Personnel Office to obtain a copy.

In anticipation of your affirmative response, we are enclosing certain personnel forms (Employee Profile Form, insurance forms, tax cards, etc.) which should be completed and returned with your acceptance. This will facilitate the timely processing of your appointment and related benefits. Also, if you have not already provided one, we will need an official copy of the academic transcript which reflects your highest earned degree; that copy should be mailed directly to us by the institution which conferred the degree.

If, as we hope, you find this offer to be satisfactory, would you please indicate your acceptance by signing, dating, and returning the original and two copies (enclosed) of this letter. A copy is enclosed for your file. An offer for which a signed acceptance is not received within 15 days of the date tendered is rescinded and becomes null and void.

We look forward to your favorable response which should be returned to the Department of Pathology together with the enclosed employment and benefits documents.

Sincerely yours,

John D. Crissman, M.D.
Professor and Chairman
Department of Pathology

Office of the Dean
School of Medicine

I accept the terms and conditions of this offer and I acknowledge receipt of a copy of the Wayne State University Personnel Manual for All Non-Represented Employees.

Gangyong Li 6-5-95

Gangyong Li, Ph.D. Date

xc: International Services Office